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Purification and characterization of three forms of glutathione transferase from *Proteus mirabilis*

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Three forms of glutathione transferase (GST) with pI values of 6.0, 6.4 and 7.3 were isolated from *Proteus mirabilis* AF 2924 by glutathione-affinity chromatography followed by isoelectric focusing, and their structural, kinetic and immunological properties were investigated. Upon SDS/polyacrylamide-slab-gel electrophoresis, all forms proved to be composed of two subunits of identical (22500) M_r. GST-6.0 and GST-6.4 together account for about 95% of the total activity, whereas GST-7.3 is present only in trace amounts. Extensive similarities have been found between GST-6.0 and GST-6.4. These include subunit molecular mass, amino acid composition, substrate specificities and immunological characteristics. GST-7.3 also cross-reacted (non-identity) with antisera raised against bacterial GST-6.0. None of the antisera raised against a number of human, rat and mouse GSTs cross-reacted with the bacterial enzymes, indicating major structural differences between them and the mammalian GSTs. This conclusion is further supported by c.d. spectra.

INTRODUCTION

Glutathione transferases (GSTs; EC 2.5.1.18) are a family of multifunctional proteins that facilitate the nucleophilic attack of GSH to a large variety of reactive electrophiles (Chasseaud, 1979; Jakoby & Habig, 1980; Mannervik, 1985). This reaction is considered to be the initial step in the formation of mercapturic acids, a pathway through which hydrophobic xenobiotics are inactivated and eliminated from the body. Furthermore, some of the isoenzymes of GST exhibit glutathione peroxidase activity towards organic hydroperoxides (Prohaska & Ganther, 1977; Di Ilio *et al.*, 1986*a*; Tan *et al.*, 1987). In addition to the catalytic function, glutathione transferases can also bind covalently/non-covalently to a wide number of hydrophobic compounds such as bilirubin, haem, drugs, carcinogens, etc. (Ketterer *et al.*, 1967; Smith *et al.*, 1977). In general, they are thought to be proteins that play a key role in detoxication processes. Cytosolic GSTs have been most extensively studied in human, rat and mouse tissues in which multiple isoforms all composed of two subunits are present (Warholm *et al.*, 1983, 1986; Tu & Reddy, 1985; Awasthi & Singh, 1985; Mannervik, 1985; Faulder *et al.*, 1987; Hayes *et al.*, 1987; Di Ilio *et al.*, 1988). These isoenzymes differ with respect to substrate specificity, subunit composition, *N*-terminal amino acid sequences, c.d. spectra, immunological properties and kinetic parameters (Mannervik *et al.*, 1985). On the basis of several criteria the considerable number of mammalian GSTs so far characterized can be grouped into three distinct classes: Alpha, Mu and Pi (Mannervik *et al.*, 1985). GSTs have also been purified from fish (Ramage *et al.*, 1986) and insects (Cochrane *et al.*, 1987), and although its activity has been detected in a number of micro-organisms (Lau *et al.*, 1980), no information is available on the chemico-physical properties of bacterial GSTs. The present paper describes the structural and immunological properties of three GST isoenzymes purified from *Proteus mirabilis* AF 2924, and compares their properties with those of mammalian tissues. Present studies also reveal that bacteria GSTs are kinetically, structurally and immunologically distinct from the GSTs characterized so far from mammalian tissues.

MATERIALS AND METHODS

Purification of glutathione transferases from *Proteus mirabilis* AF 2924

Washed cells of *Proteus mirabilis* AF 2924 (Type Culture Collection of the Institute of Experimental Medicine, Chieti, Italy) were grown aerobically for 18 h at 37 °C in Trypticase Soy Broth (BBL Microbiology Systems; Becton–Dickinson, Cockeysville, MD 21030, U.S.A.), suspended in 10 mM-potassium phosphate buffer, pH 7.0 (buffer A), and disrupted by sonication (five bursts of 3 min each, at 300 W) with a Labosonic 1510 (Braun) sonicator. The particulate material was removed by centrifugation at 105000 *g* for 1 h and the supernatant applied to a GSH–Sepharose affinity column (1 cm × 10 cm) (Simons & Vander Jagt, 1987) which was pre-equilibrated with buffer A. The column was exhaustively washed with buffer A supplemented with 50 mM-KCl. The enzyme was eluted with 50 mM-Tris/HCl buffer, pH 9.6, containing 5 mM-GSH. The fractions showing GST activity were pooled, concentrated by ultrafiltration, dialysed against buffer A and subjected to isoelectric focusing on a column (110 ml; LKB Produkter, Stockholm, Sweden) containing 1% Ampholine pH 3.5–10 plus 1% Ampholine pH 9–11 in a 0–40%-(w/v)-sucrose density gradient. After focusing for 72 h at a final voltage of 700 V (4 °C), the content of the column was eluted and collected in 1.2 ml fractions.

Abbreviations used: GST, glutathione transferase; GST-6.0 etc., GST with a pI value of 6.0 etc.; PAGE, polyacrylamide-gel electrophoresis.

The three peaks of activity thus separated were concentrated by ultrafiltration, dialysed against buffer A and used for further characterization. All operations in the purification procedure were performed at 4 °C.

SDS/polyacrylamide-gel electrophoresis (PAGE)

SDS/electrophoresis PAGE in discontinuous slab gels was performed by the method of Laemmli (1970). The SDS concentration was 0.1% (w/v), and the spacer and the separating gels contained 3% and 12.5% acrylamide respectively. Bovine serum albumin (M_r 66000), ovalbumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000), trypsinogen (M_r 24000), soybean trypsin inhibitor (M_r 20100), α-lactalbumin (M_r 14200) were used as standards for characterization of subunit molecular size. The M_r of the native enzyme was determined by gel filtration on a column (1.5 cm × 70 cm) of Sephadex G-100 equilibrated with 100 mM-phosphate buffer, pH 6.7. Bovine serum albumin (M_r 66000), human placental GST (M_r 46000), chymotrypsinogen A (M_r 25000), myoglobin (M_r 17000) and lysozyme (M_r 14000) were used as molecular-size markers.

Amino acid analysis

Amino acid composition was determined on protein samples precipitated with trichloroacetic acid as described by Marcus *et al.* (1978). Hydrolysis was performed in 6 M-HCl at 110 °C for 48 h in evacuated sealed tubes. The HCl was removed under vacuum, and the samples dissolved in citrate buffer were applied to an LKB Alpha amino acid analyser. Compositions are based on an M_r of 45000.

Immunological studies

Antibodies against *Proteus mirabilis* GST-6.0 were raised in rabbits via two injections of 100 μg of protein in Freund's complete adjuvant. Antisera against mouse liver GSTs MI and MII, against human uterus GST III, GST V, against human placenta GST-π, as well as against GST-8.5 of human skin were available in our laboratory and were the same as those used in previous studies (Di Ilio *et al.*, 1986*b*, 1987, 1988). Antisera against rat GST 1-1, 2-2, 7-7 and 8-8 were purchased from Bioprep (Dublin, Ireland). Ouchterlony immunodiffusion experiments were performed at 4 °C in 1%-(w/v)-agarose gels containing 50 mM-potassium phosphate buffer, pH 6.9, and 0.9% NaCl.

Enzyme assay

GST activity with the various substrates was assayed at 24 °C in a Beckman model 3600 spectrophotometer according to the methods of Habig & Jakoby (1981). The Se-independent glutathione peroxidase activity of GST was measured with cumene hydroperoxide as previously reported (Di Ilio *et al.*, 1986*a*). Protein concentration was determined by the method of Bradford (1976), with γ-globulin as standard.

C.d. spectra

C.d. spectra were obtained with a Jasco J-500 A instrument equipped with a Jasco DP-500 N data processor. The molar ellipticity (degrees·cm^2·dmol^{-1}) is expressed as $[\theta]_M$ on a molar protein basis in the 250–320 nm range and as a mean residue ellipticity ($[\theta]_{m.r.w.}$) in the 200–250 nm range (mean residue M_r 115). The content of secondary structure was estimated from the c.d. spectra between 200 and 250 nm as described by Greenfield & Fasman (1969).

RESULTS

Purification

Table 1 summarizes the results of a typical purification of GST isoenzymes present in *P. mirabilis* cytosol using 1-chloro-2,4-dinitrobenzene as substrate. Owing to the turbidity of the solution the measurement of activity in the 105000 *g* supernatant was not possible, but a value of 0.8 unit/mg after affinity chromatography on GSH–epoxy-activated-Sepharose was found. No measurable activity passed straight through the affinity matrix. After affinity chromatography of the bacterial cytosol, three forms of the enzyme, with a pI values of 6.0, 6.4 and 7.3, were resolvable by isoelectric focusing in 3.5–10 pH range (Fig. 1). The same pI values were obtained when the enzymes were subjected to re-isoelectric focusing separately. We have designated these isoenzymes as bacterial GST, suffixed with their isoelectric point.

Table 1. Purification of GST from *Proteus mirabilis*

Step	Specific activity (μmol/min per mg)	Total activity (μmol/min)	Total protein (mg)	Yield (%)
Cytosol	–	–	13000	–
Affinity chromatography	0.79	6.2	7.8	100
Isoelectric focusing				
GST-6.0	3.0	4.5	1.5	72
GST-6.4	1.4	1.54	1.1	25
GST-7.3	0.16	0.05	0.3	0.8

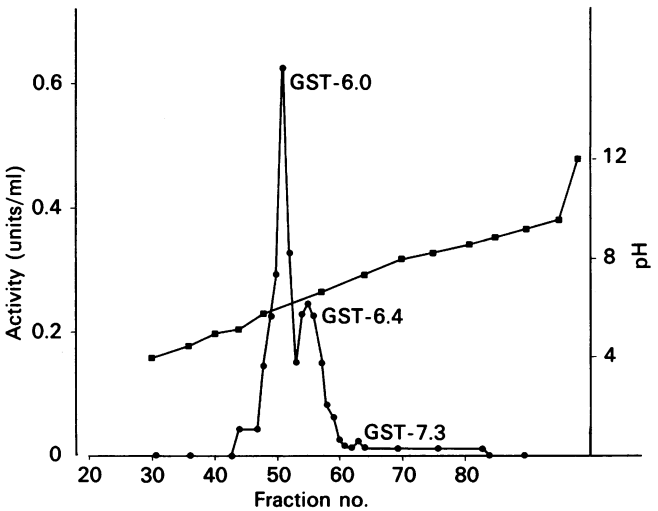

Fig. 1. Separation of cytosolic GSTs from *Proteus mirabilis* by isoelectric focusing in a 110 ml column

Fractions (1.2 ml each) were collected, and the enzymic activity with 1-chloro-2,4-dinitrobenzene (●) as well as the pH (■) were measured.

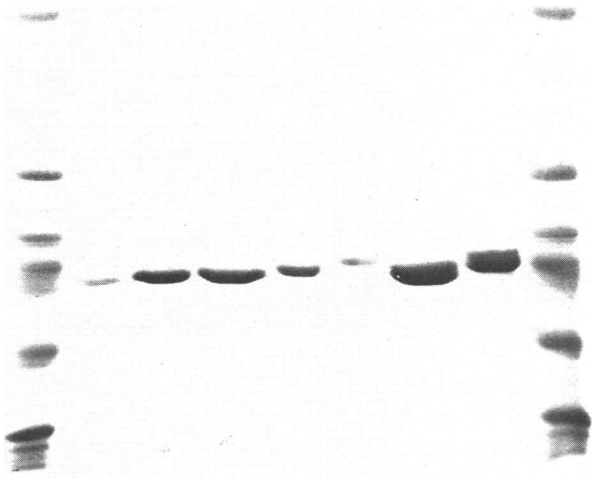

Fig. 2. Slab SDS/PAGE of the purified isoenzymes of the GSTs of *Proteus mirabilis*

Lanes 1 and 9, standards and their M_r values, from top to bottom, are: bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), soybean trypsin inhibitor (20100), α-lactalbumin (14200 M_r); lane 2, GST-7.3; lane 3, GST-6.4; lane 4, GST-6.0; lane 5, GST-π; lane 6, human kidney GST-8.7; lane 7, mouse GST MII; lane 8, mouse GST MI.

Structural studies

Size-exclusion chromatography of bacterial GSTs gave a M_r value of about 48000 (results not shown). The relative electrophoretic mobility on SDS/PAGE of individual GSTs can be seen in Fig. 2. The three peaks obtained from isoelectric focusing produced a single band, indicating that all the fractions were free of contaminating proteins. Compared with proteins of known molecular size, their M_r values were estimated to be 22500. The electrophoretic mobilities on SDS/PAGE of bacterial GSTs were also compared with the anionic GST (GST-π) of human placenta (Di Ilio *et al.*, 1986*b*), with the cationic GST (GST-8.7) of human kidney (Di Ilio *et al.*, 1987) as well as with the MI and MII GST isoenzymes of mouse liver. It is evident from Fig. 2 that bacterial GSTs migrate slightly faster than human anionic GST-π.

Substrate specificities

Table 2 lists the specific activity, of each of the bacterial isoforms isolated, for various substrates. GST-7.3 was active with 1-chloro-2,4-dinitrobenzene only, and no conjugation activity was recorded with the other substrates listed in Table 2 in the presence of 300 μg of purified protein. The activity towards the standard and most highly reactive substrate of mammalian GSTs, namely 1-chloro-2,4-dinitrobenzene, was relatively low. GST-6.0 and GST-6.4 exhibited remarkably similar substrate specificities. Of note is the higher activity with *trans*-4-phenyl-3-buten-2-one of GST-6.4. Moderate, yet significant, activity was noted with both cumene hydroperoxide and ethacrynic acid. No activity was obtained with bromosulphophthalein. It is noteworthy that bacterial glutathione transferases are able to reduce cumene hydroperoxide and to conjugate ethacrynic acid, which are diagnostic substrates for mammalian GST isoenzymes belonging to distinct classes (Mannervik, 1985).

Table 2. Specific activity of GSTs of *Proteus mirabilis* toward different substrates

Abbreviation: ND, no detectable activity.

	Specific activity (μmol/min per mg)		
Substrate Isoenzyme...	GST-6.0	GST-6.4	GST-7.3
1-Chloro-2,4-dinitrobenzene	3.0	1.4	0.16
Ethacrynic acid	0.055	0.051	ND
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	0.04	0.025	ND
trans-4-Phenyl-3-buten-2-one	0.0008	0.002	ND
Bromosulphophthalein	ND	ND	ND
Cumene hydroperoxide	0.75	0.62	ND

Immunological properties

Attempts to prepare antisera against the individual isoenzymes of bacteria were only successful for GST-6.0. In immunodiffusion experiments, antisera raised against GST-6.0 cross-reacted with both GST-6.4 (complete identity) and GST-7.3 (partial identity as indicated by spur formation) (Fig. 3). These results show that GST-6.0 and GST-6.4 have identical antigenicity, which is similar to, but not identical with, the antigenicity of GST-7.3. The immunological relationships between bacterial and mammalian GSTs were also studied by performing Ouchterlony double-diffusion experiments with the antisera raised against GST V and GST III of human uterus (Di Ilio *et al.*, 1988), GST pI 8.5 (Del Boccio *et al.*, 1987) of human skin and human placenta GST-π (Di Ilio *et al.*, 1986*b*), with the antisera raised against mouse GSTs MI and MII (Warholm *et al.*, 1986) and with the antisera raised against rat GSTs 1-1, 2-2, 7-7 and 8-8. No precipitin lines were detected between these antisera and *P. mirabilis* GST-6.0, GST-6.4 and GST-7.3. Conversely, identical results were obtained when antisera raised against bacteria GST-6.0 was tested with human, rat and mouse isoenzymes.

Amino acid composition

The amino acid compositions of the three bacterial isoenzymes are reported in Table 3. The amino acid analysis of the isoenzymes showed that they possessed almost identical amino acid compositions. In order to assess the amino acid compositional similarities among the bacterial and mammalian enzymes, the difference index of Metzger *et al.* (1968) was calculated. Values of 7 and 5.9 were estimated when GST-6.0 was compared with GST-6.4 and GST-7.3 respectively, whereas a value of 5.6 was obtained comparing GST-6.4 and GST-7.3. A comparison of bacterial and mammalian GSTs of class Alpha, Mu and Pi gives values higher than 16 (Di Ilio *et al.*, 1988; Mannervik, 1985; Del Boccio *et al.*, 1987; Warholm *et al.*, 1986).

C.d. spectra

Fig. 4(*a*) and Fig. 4(*b*) show the c.d. spectra, in the near- and far-u.v. regions respectively, of purified

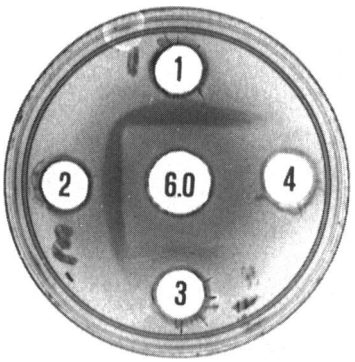

Fig. 3. Ouchterlony double-diffusion analysis of GSTs isolated from *Proteus mirabilis*

The antisera raised against bacterial GST-6.0 ('6.0') was placed in the centre well and bacterial GST-6.4 (1), GST-6.0 (2), GST-7.3 (3) and GST-π (4) were placed in the peripheral wells as indicated. The precipitin lines were stained with Coomassie Brilliant Blue R-250.

Table 3. Amino acid composition of glutathione transferases of *Proteus mirabilis*

Amino acid analyses were carried out after hydrolysis at 110 °C in 6 M-HCl for 48 h. Compositions are based on an M_r of 45000.

		Composition (residues/molecule)		
Amino acid	Isoenzyme...	GST-6.0	GST-6.4	GST-7.3
Aspartic acid		30.8	22.2	34.3
Threonine		21.6	19.4	17.9
Serine		26.6	30.6	23.6
Glutamic acid		39.8	41.3	36.4
Proline		24.8	21.3	19.0
Glycine		23.1	27.1	21.8
Alanine		25.6	25.4	22.8
Valine		25.8	21.3	23.0
Cystine		2.9	2.0	2.1
Methionine		1.3	2.4	2.6
Isoleucine		20.0	16.0	16.5
Leucine		42.7	39.4	42.8
Tyrosine		16.4	14.8	14.6
Phenylalanine		14.5	12.9	12.3
Histidine		15.2	13.8	14.5
Lysine		30.3	29.3	29.2
Arginine		34.3	49.0	48.7

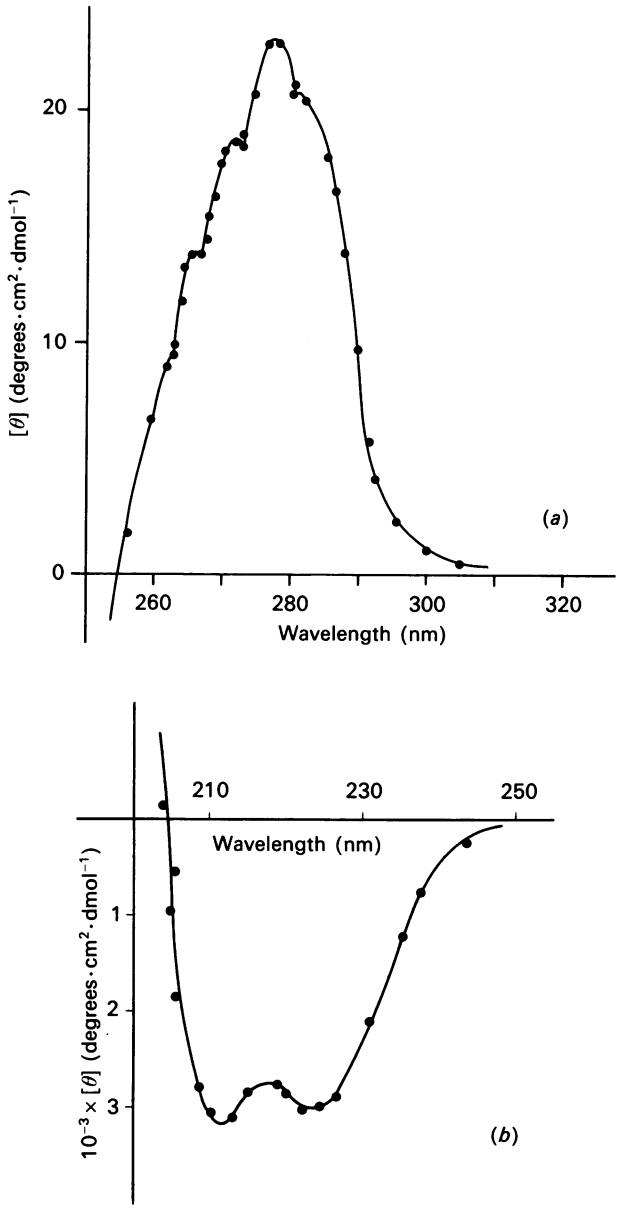

Fig. 4. C.d. spectra of purified bacterial GST-6.0 (*a*) between 250 and 320 nm and (*b*) between 200 and 250 nm

Molar ellipticity was calculated from a mean residual weight of 115. The spectra were recorded in 0.01 M potassium phosphate buffer, pH 6.5.

bacterial GST-6.0. Bands probably originating from tryptophan side chains are seen at 277 nm and at 284 nm. From the c.d. spectra in the low-u.v. region shown in Fig. 4(*b*), the secondary structure of purified bacteria GST-6.0 was calculated. From the amplitude of the two negative peaks (at 224 nm and 211 nm) an α-helical content of about 5% can be estimated (Greenfield & Fasman, 1969). These results clearly indicate that the c.d. spectra of bacterial and mammalian (Di Ilio *et al.*, 1988; Warholm *et al.*, 1983; Maruyama *et al.*, 1984) GSTs are completely different, confirming that they have major structural differences.

DISCUSSION

With the use of standard techniques, i.e. affinity chromatography and isoelectric focusing, at least three isoenzymes of GST with isoelectric points at pH 6.0, 6.4 and 7.3 can be resolved from *Proteus mirabilis*. The two major GSTs, designated here as 'GST-6.0' and 'GST-6.4' account for about 70 and 25% of activity respectively, whereas GST-7.3 is present only in limited amounts. The different forms of GSTs together constitute about 0.06% of the total cytosolic proteins. This value is lower than the value (3–5%) found for GSTs of mammalian tissues (Chasseaud, 1979; Jakoby & Habig, 1980; Mannervik, 1985). GSTs GST-6.0, GST-6.4 and GST-7.3 all proved

to be dimers of identical M_r (22500). This is in contrast with rat GSTs, which include heterodimeric proteins in addition to homodimers (Mannervik, 1985*a*; Awasthi & Singh, 1985; Hayes *et al.*, 1987). GST-6.0 and GST-6.4 appear quite similar in many respects. This is indicated by similarities of subunits M_r, amino acid composition, substrate specificities and, especially, of their immunological properties. These results suggest that the two proteins are conformational isomers with different net charges. The apparent similarities between the two isoenzymes also suggest the possibility that the two forms may have been arisen by post-translational modification of a single precursor. GST-7.3 also seems structurally related to other two bacterial GSTs; however, when GST-6.0 and GST-7.3 were tested in parallel against anti-(GST-6.0) serum, the enzymes gave a precipitin line that merged with spurs, indicating that the epitopes of the two proteins are similar, but not identical. Bacterial GSTs characterized herein demonstrate similarities with those of other species, in that the subunit M_r values fall within the ranges described for mammalian GSTs. In addition, the range of substrates utilized, although narrower, is reminiscent of that of mammalian enzymes. The bacterial GSTs are similar to certain rat and human isoenzymes in exhibiting a relatively high glutathione peroxidase activity (Mannervik, 1985). Bacterial GSTs appear, however, structurally different from mammalian enzymes, as indicated by immunological studies, amino acid compositions and c.d. spectra. It is particularly noteworthy that none of the antibodies raised against a large number of human, rat and mouse GSTs cross-reacted in immunodiffusion experiments with the three forms isolated from the bacterium. This indicates that the antigenic domain of bacterial GSTs is different from those of mammalian origin. Thus bacterial GSTs are kinetically, structurally and immunologically unlike mammalian isoenzymes thus far characterized. Mannervik *et al.* (1985) have grouped the mammalian GSTs into three distinct groups: Alpha, Mu and Pi. The results of the present investigation seem to indicate that this classification cannot be extended to bacterial GSTs.

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